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ORIGINAL PAPER

Mixture of sugar and povidone-iodine stimulates healing of MRSA-infected skin ulcers on *db/db* mice

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Abstract The topical application of a mixture of sugar and povidone-iodine (PI) has been reported to accelerate the healing of cutaneous wounds and ulcers by promoting reepithelialization and granulation tissue formation, as well as by having an anti-microbial effect. In order to clarify the efficacy of a 70% sugar and 3% PI paste (U-PASTA™)(SP) on infectious skin ulcers, we made a bacterial infection model using methicillin-resistant *Staphylococcus aureus* (MRSA) on the skin of diabetic *db/db* mice, and investigated the effect of the paste on the healing process of wounds. Full-thickness wounds were made on the backs of female diabetic mice, (C57BL/ksJ *db/db*) and inoculated with *S. aureus*. SP was applied to the closed wounds for 8 days. The degree of repair was evaluated using three histological parameters: The degree of reepithelialization was given a percentage value of 0–100%; the amount of granulation tissue was quantified by measuring the area of granulation (mm^2); and the number of capillary lumens in the granulation tissue was counted in the complete wound cross-section at 100× magnification. In addition, the colony-forming units (CFU) of MRSA on the wounds were counted. Continuous MRSA infection in the wounds of *db/db* mice was demonstrated with macroscopic and histopathological images. Wounding and infection caused by MRSA on the back of the diabetic mice significantly induced delayed reepithelialization, granulation tissue formation with inflammatory cell infiltrate and increased CFU on wounds ($P < 0.01$, respectively) compared to those of the MRSA-infected normal mice. Application of SP significantly accelerated reepithelialization ($P < 0.01$) and decreased CFU ($P < 0.05$) of the ulcers in the MRSA-infected wounds, compared to the non-treated group. Histopathological evaluation and CFU on this animal model revealed no significant difference between Methicilin-sensitive *Staphylococcus aureus* and MRSA infection. These results indicate that wounding on *db/db* mice provides a useful animal model of bacterial skin infections, and that SP is an effective topical agent for the treatment of diabetic skin ulcers.

Keywords Topical agent · Animal model · Diabetes mellitus · MRSA

Abbreviations

PI	Povidone-iodine
SP	A paste comprising 70% sugar and 3% PI
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicilin-sensitive *Staphylococcus aureus*
CFU	Colony-forming units
PBS	Phosphate buffered saline

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Introduction

Various animal models of infected wounds have been reported in the past, including those using mice [23], rats [12, 24] and pigs [6]. The speed of wound healing and the anti-bacterial function of normal animals are generally excellent, so that most of the observation periods for reported models were only 10 days or less. The wounds in such models were merely transient traumatic ulcers with only a superficial presence of bacteria, but not a stable infection. We cannot evaluate the anti-bacterial effect of topical agents in vivo unless we adopt animal models, which actually have some form of chronic bacterial infection. Although the precise interactions of the components comprising normal wound healing are not understood, a number of conditions such as immunodeficiency, chemotherapy, venous stasis, and diabetic ulcers result in delayed wound healing [8]. We reported an animal model using healing-impaired diabetic mice, which was a very useful tool for evaluating the wound healing effects of topical agents [26–28]. Since there were no appropriate animal models of chronically infected wounds, we prepared a methicillin-resistant *Staphylococcus aureus* (MRSA) infection model of the skin by utilizing diabetic *db/db* mice.

Povidone-iodine (PI), a compound of iodine and polyvinylpyrrolidone, is a common anti-microbial agent [16] and has been used as a surgical scrub or a skin cleanser in various forms. The use of PI as a topical healing-stimulatory agent was limited because of its toxicity [7]. In Europe and North America as well as Japan, Cadexomer-iodine is the commercially available ointment, which releases iodine slowly from beads of dextrin and epichlorhydrin. This preparation is an effective debridement and antiseptic agent for treating chronic exudative wounds [5, 9, 29], removing necrotic tissue, bacterial components and biofilm synthesized by bacteria [2, 3], and showing stimulation of epidermal regeneration [15]. A liposomal hydrogel containing 3% PI, another commercially available topical reagent, successfully cleans the wound, and enhances wound closure [19]. Sugar and its related products from various natural sources have been used to promote wound healing [13]. Sugar promoted reepithelialization and granulation tissue formation in normal full-thickness wounds in rats [10]. Some of the mechanisms thought to underlie sugar-promoted wound healing are the osmotic effect, which prevents bacterial growth [1] and the acceleration of granulation tissue formation partly by the mechanical cleansing of necrotic tissue in wounds [11]. Knutson et al. [14] have reported in a clinical study that a compound consisting of 70–80% sugar and PI solution remarkably enhanced healing and reduced bacterial contamination in a wide variety of wounds, burns and ulcers. Similarly, topical application of the mixture of sugar and PI successfully treated diabetic and burn ulcers [4, 25]. A paste consisting of 70% sugar and 3% PI has been commercially available in Japan (U-PASTA™, Kowa company Ltd, Nagoya, Japan). It is reported that U-PASTA is clinically effective in promoting rapid healing of wounds and reducing bacterial contamination [17, 18, 22]. U-PASTA also influenced the migration of keratinocytes and fibroblasts through the pharmaceutical effects of PI on these cells, while stimulating the increase of granulation tissues mainly through the effects of sugar in vitro [20].

In order to clarify the efficacy of a 70% sugar and 3% PI paste (U-PASTA) (SP) on the healing process in infectious skin ulcers, we prepared a bacterial infection model on the skin of diabetic *db/db* mice for use with the paste in combination with MRSA representing several kinds of multidrug-resistant bacteria.

Materials and methods

Reagents

A paste (SP) consisting of 70% sugar and 3% PI (US patent 4844898, U-PASTA) manufactured by Kowa Company Ltd (Nagoya, Japan). SP was developed in answer to a need for a substance, which was chemically stable, physically homogeneous and spreadable, unlike a simple mixture of sugar and PI. SP contains a water soluble base including polyethylene glycol 400, glycerin and water, in addition to sugar and PI.

Animals and wounding

Wounding design and sampling for histological assessment were performed according to our previously reported methods [26]. Female mutant diabetic mice (C57BL/ksJ *db/db*) and heterozygous control mice (C57BL/ksJ *db/+*), were purchased from Jackson Laboratory (Bar Harbor, ME, USA). All mice were housed individually and maintained on a standard laboratory diet and received water ad libitum. Mice at 8 weeks of age were anesthetized with sodium pentobarbital solution (25 mg/kg, injected intraperitoneally, Abbott Laboratories, North Chicago, IL, USA) and their dorsal hair was gently clipped. Two round, full-thickness wounds were prepared on the back of each mouse in the anterior-posterior direction using a punch biopsy instrument (3 mm diameter, Maruho Co., Osaka, Japan). The wounds applied with SP were covered with a sterilized transparent dressing (Cathereep, Nichiban Co., Tokyo, Japan), and the mice were sacrificed at day 13 by cervical dislocation. The wounds were excised and fixed in 10% buffered formalin solution.

Microorganism

Methicillin-resistant *Staphylococcus aureus* (N315 PZR) and Methicilin-sensitive *Staphylococcus aureus* (MSSA) (N315 P) were kindly donated by Prof. Keiichi Hiramatsu, Department of Microbiology, Juntendo University School of Medicine. The stocks had been stored at −70°C. A working stock culture was obtained by culture in an aliquot on 0.5% sheep blood agar and trypticase agar. One day before wounding, a single colony of MRSA or MSSA was taken from the stock plate with a sterile loop, inoculated into trypticase soy broth, and incubated for 24 h at 37°C. The culture was then centrifuged, and the supernatant was discarded. Then the bacterial pellet was resuspended in phosphate buffered saline (PBS) at a concentration of 1×10^5 organisms per 30 µl.

Inoculation of *S. aureus* to the animals and counting of colony-forming units

Female mutant diabetic mice (C57BL/ksJ *db/db*) at 8 weeks of age were anesthetized and their dorsal hair was gently clipped. Two round, full-thickness wounds 3 mm in diameter were prepared on the back of each mouse in the anterior–posterior direction as described previously. Each wound was covered with a sterilized transparent dressing (Cathereep, Nichiban Co.) and inoculated with 1×10^5 MRSA (N315 PZR) or MSSA (N315 P) in 30 µl of PBS, or buffer alone, by injection into the wounds covered by Cathereep (Nichiban). The animals were sacrificed on day 8, day 13 or day 19. For the evaluation of the effect of SP on infected wounds, each 3 mm wound was inoculated with 1×10^5 MRSA (N315 PZR) in 30 µl of PBS, and covered with Cathereep (Nichiban) for 3 days. After the visual confirmation of redness and pus on wounds, SP was applied four times every other day. The wounds treated with SP were covered with Cathereep, and the mice were sacrificed at day 13 by cervical dislocation. Bacterial cultures on the wounds were established and colony-forming units (CFU) were counted, then the histopathological specimens were prepared. In orderto count CFU, each infected wound surface was scrubbed carefully using a sterile swab, which was soaked in 1 ml of PBS. The solution was then diluted by the tenfold dilution method and cultured on 0.5% sheep blood agar and trypticase agar. All of the experiments were performed in a negatively pressurized animal house in filtered air at Kowa Research Institute.

Histological evaluation

After fixation overnight, the tissue was trimmed and cut through at the widest margin. The tissue was embedded in paraffin and sliced into 5-µm sections. The sections were made perpendicular to the anterior–posterior axis and to the surface of the wound. Three sections were placed on a slide, and stained with hematoxylin and eosin (HE). Some specimens of MRSA-infected wounds were observed with Gram staining, and the infection was confirmed. The section with the widest original wound margin was used for assessment. The parameters measured were degree of reepithelialization, area of granulation tissue and number of capillaries. Each of the parameters was graded numerically as described below.

Reepithelialization

The degree of reepithelialization was measured by a computerized morphometric analysis (IBAS-2000, Zeiss, Germany) and was given a percentage value, 0% being equivalent to no closure and 100% equivalent to complete wound closure.

Area of granulation tissue

The amount of granulation tissue was quantified by measuring the area of granulation tissue (mm^2) in the section perpendicular to the surface of the wound. Granulation tissue was traced by computerized morphometric analysis (IBAS-2000).

Capillary number

The number of capillary lumens in the granulation tissue was counted in the complete wound cross-section at 100× magnification.

Statistical analysis

The data obtained were compared with the results of other groups using the Student's unpaired *t*-test. Results were expressed as mean values ± standard error of means (SEM).

Results

MRSA infection to the diabetic mice delayed the wound healing

Two round, full-thickness wounds 6 mm in diameter were prepared on the back of 20 female mutant diabetic mice (C57BL/ksJ *db/db*) and 20 heterozygous control mice (C57BL/ksJ *db/+*). Each wound on the ten mice in each group was covered and inoculated with 1×10^5 MRSA (N315 PZR) (Fig. 1d) for 19 days. The non-inoculated wounds (Fig. 1a) were also covered with the dressing for 13

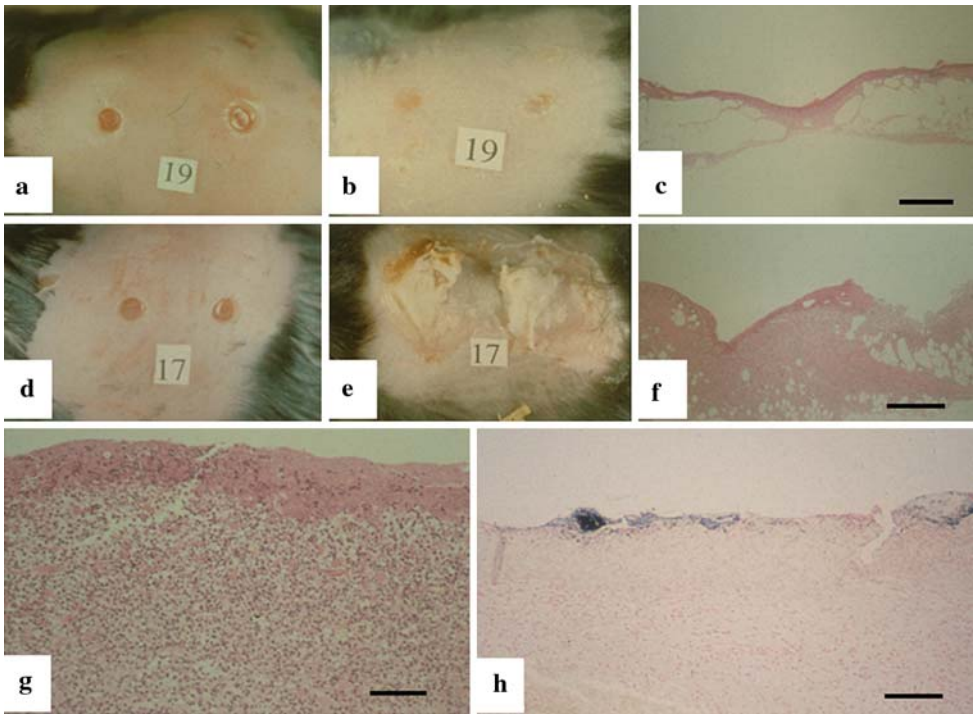

Fig. 1 Wounding and MRSA infection of C57BL/ksJ *db/db* mice. Two round, full-thickness wounds 3 mm diameter were prepared on the back of each C57BL/ksJ *db/db* mouse in the anterior-posterior direction, and infected by 1×10^5 MRSA (N315 PZR). **a** C57BL/ksJ *db/db* mice without MRSA infection at day 0. **b** C57BL/ksJ *db/db* mice without MRSA infection at day 13. **c** Histopathology of the wound in panel "b" (HE staining). **d** C57BL/ksJ *db/db* mice with MRSA infection at day 0. **e** C57BL/ksJ *db/db* mice with MRSA infection at day 19. **f** Histopathology of the wound in panel "**e**" (HE staining). **g** High magnification of panel "**f**" focusing on inflammatory infiltrate (HE staining). **h** Gram staining of adjacent serial section of panel "**f**". (**c**, **f**) *Scale bars*: 500 μm. (**g**, **h**) *Scale bars*: 100 μm

days because most of the non-infected wounds had reepithelialized by day 13. The animals were sacrificed on the last day. Histopathological specimens were prepared, and CFU on the wounds were counted. The non-treated wounds of diabetic mice had reepithelialized (Fig. 1b, c), whereas the MRSA-infected wounds of diabetic mice were purulent (Fig. 1e, f). HE staining of the wounds of each group revealed delayed reepithelialization (Fig. 1f), inflammatory cell infiltration and edema (Fig. 1g) due to MRSA infection. Gram staining of MRSA-infected wounds showed numerous Gram positive cocci (Fig. 1h).

The course of reepithelialization and granulation tissue formation is shown in Fig. 2. The reepithelialization process of MRSA-infected wounds in diabetic mice (open circle) was delayed compared to those of non-treated diabetic mice (open square), normal mice with infection (closed circle) and normal mice without infection (closed square) (Fig. 2a). Granulation tissue formation was increased in MRSA-infected wounds of both diabetic mice (open circle) and normal mice (closed circle) compared to the non-treated wounds of diabetic mice (open square) and normal mice (closed square), respectively (Fig. 2b). According to the statistical analysis at day 13 (Fig. 3), reepithelialization in MRSA-infected *db/db* mice (Fig. 3a) was significantly decreased [$22 \pm 8\%$ ($n = 10$) vs $99 \pm 1\%$ ($n = 7$), $P < 0.01$], while granulation tissue formation [2.9 ± 0.3 mm^2 ($n = 10$) vs 1.5 ± 0.3 mm^2 ($n = 7$), $P < 0.01$, Fig. 3b] and CFU of MRSA [447 ± 42 ($n = 10$) vs 100 ± 55 ($n = 7$), $P < 0.01$, Fig. 3c] were significantly increased compared to those of normal groups.

Infection in an ulcer means that the inoculated bacteria have proliferated in the lesion, erythema, exudation and pustules can be observed macroscopically, numerous bacteria are cultured bacteriologically, and abscesses and bacterial components can be found in the ulcer histopathologically. We confirmed these factors macroscopically and histopathologically (Fig. 1), as well as confirming CFU (Fig. 3c). These results demonstrate the usefulness of this animal model of infectious skin ulcers.

Effects of SP on the animal model of MRSA infection

Two round, full-thickness wounds 3 mm in diameter were prepared on the back of ten female mutant diabetic mice, and inoculated with 1×10^5 MRSA (N315 PZR). After confirmation of MRSA infection on day 3, SP was applied to the ten wounds on each of the five animals four times every other day. For the control group, consisting also of

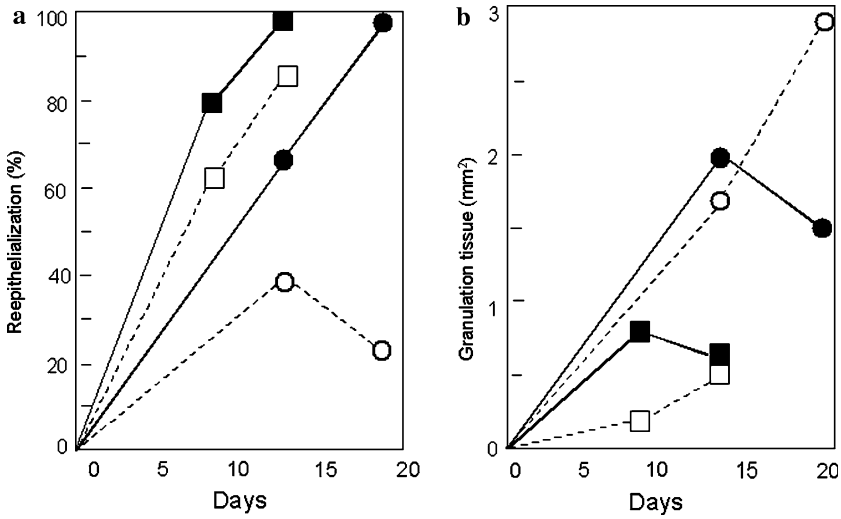

Fig. 2 The course of wound healing in reepithelialization and granulation tissue formation in diabetic and normal mice. **a** Reepithelialization. **b** Granulation tissue formation. MRSA-infected wounds of both diabetic mice (*open circle*) and normal mice (*closed circle*), and the untreated wounds of diabetic mice (*open square*) and normal mice (*closed square*) are shown in each panel

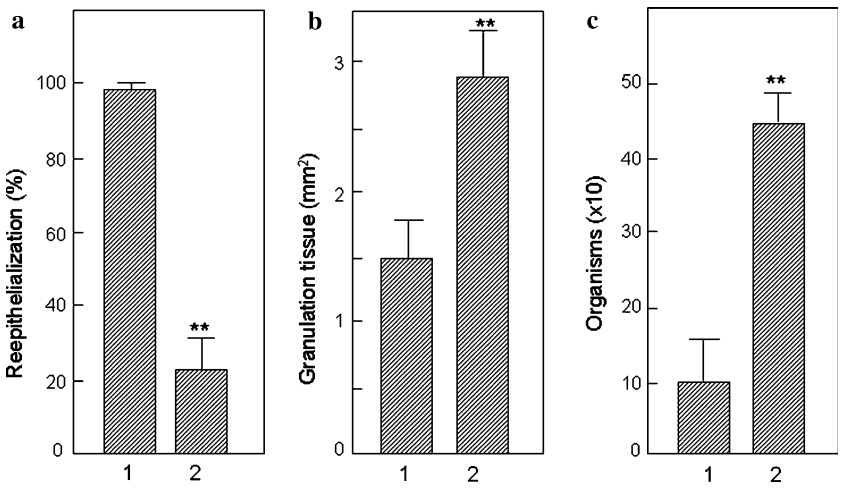

Fig. 3 Wound healing in diabetic and normal mice with MRSA infection. **a** Reepithelialization. **b** Granulation tissue. c Organisms. *Lane 1*: C57BL/ksJ *db/+* mice, $n = 7$. *Lane 2*: C57BL/ksJ *db/db* mice, $n = 10$. Data are expressed as the mean + SEM. $^{**}P < 0.01$ compared with the control group (*lane 1*)

five animals, each with ten wounds, the transparent dressing (Cathereep) was changed every other day and no reagents were applied. Some of the animals died during the experiment because of problems due to anesthesia or sepsis; others scratched and removed the dressings. We finally chose eight wounds treated with SP and five untreated wounds for evaluation. Bacterial cultures from the wounds were performed, and histopathological specimens were prepared. Then the four parameters, namely, degree of reepithelialization, area of granulation tissue, number of capillaries and CFU of MRSA, were measured or counted. The results (Fig. 4) showed that reepithelialization on wounds treated with SP had accelerated significantly [$95 \pm 5\%$ ($n = 8$) vs $24 \pm 19\%$ ($n = 5$), $P < 0.01$, Fig. 4a] while CFU of MRSA on the wounds had decreased significantly [$1.1 \pm 0.6 (n = 8)$ vs $126 \pm 42 (n = 5)$, $P < 0.05$, Fig. 4d] compared with the non-treated group. Although there was no difference in granulation tissue area between the two groups, the granulation tissue of SP-treated wounds was compact whereas untreated tissue was edematous (Fig. 5). Application of SP decreased the number of capillaries but only to a statistically insignificant degree (Fig. 4c). An image of typical tissue is shown in Fig. 5. The wounds treated with SP were closed and compact granulation tissue was evident, whereas infiltration of polymorphonuclear leukocytes, abscesses, thick encrustation and delay in reepithelialization were observed in the untreated wounds.

Comparison of MSSA and MRSA infection on this model

Two round, full-thickness wounds 3 mm in diameter were prepared on the back of female mutant diabetic mice, inoculated with 1×10^5 MRSA (N315 PZR) or MSSA (N315P), and covered with a sterilized transparent dressing (Cathereep) for 13 days. The animals were sacrificed on the last day. Histopathological specimens were prepared, and CFU on the wounds were counted.

The results indicated that reepithelialization of MSSA- and MRSA-infected wounds had decreased significantly

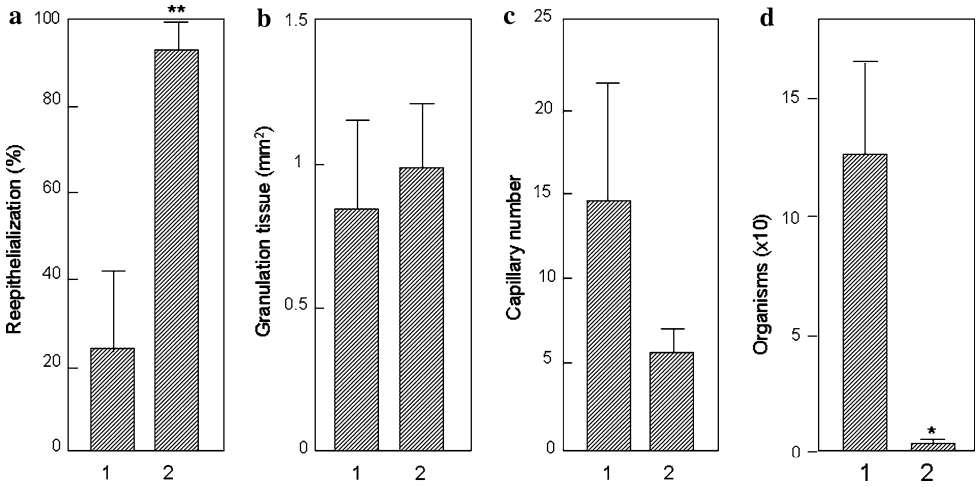

Fig. 4 Effects of SP on the animal model of MRSA infection. **a** Reepithelialization. **b** Granulation tissue. **c** Capillary number. **d** CFU of MRSA. *Lane 1*: no treatment, $n = 8$. *Lane 2*: SP, $n = 5$. Data are expressed as the mean + SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control group (*lane 1*)

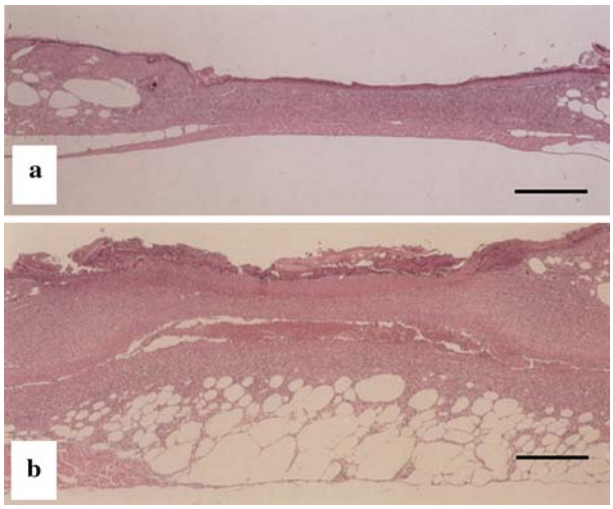

Fig. 5 Histopathological images of wounds infected with MRSA with/without SP treatment. **a** Treated with SP. **b** Untreated (HE staining, *scale bar*: 500 μm)

[$40 \pm 5\%$ ($n = 15$) and $30 \pm 7\%$ ($n = 14$) vs $91 \pm 5\%$ ($n = 10$), $P < 0.01$, Fig. 6a], while granulation tissue formation had increased significantly [$2.44 \pm 0.31 mm^2$ ($n = 15$) and $1.92 \pm 0.24\%$ ($n = 14$) vs $0.57 \pm 0.06\%$ ($n = 10$), $P < 0.01$, Fig. 6b]. The number of colonies of these organisms had also increased significantly [263 ± 62 ($n = 16$) and 244 ± 75 ($n = 16$) vs 0.14 ± 0.14 ($n = 14$), $P < 0.01$, Fig. 6C] compared with the non-treated group (lane 1 in each panel). The wounds infected with MSSA did not reveal any significant difference from those infected with MRSA in terms of the three parameters, namely, reepithelialization, granulation tissue formation and CFU (Fig. 6).

Discussion

In order to determine the anti-bacterial, as well as wound healing, effects of SP, we established a chronic infection model in the present study. Wounding and infection of MRSA on the back of diabetic mice (C57BL/ksJ *db/db*) induced continuous bacterial infection, inflamatory cell infiltration and delayed reepithelialization, which mimic chronic bacterial infections in human skin. Even when MRSA was inoculated on the wounds of normal mice, reepithelialization proceeded and the organisms were rejected (Figs. 2, 3). Histological evaluation of wound healing

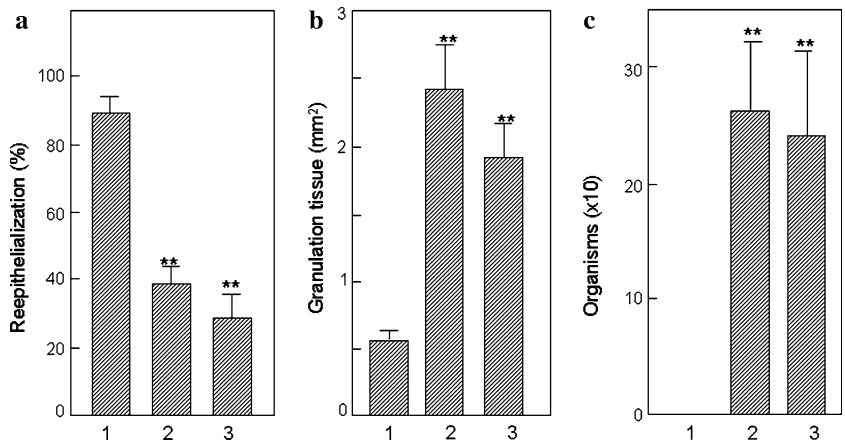

Fig. 6 Methicilin-sensitive *Staphylococcus aureus* and MRSA infection in animal model. **a** Reepithelialization. **b** Granulation tissue. **c** Numbers of organisms. *Lane 1*: No treatment. *Lane 2*: MSSA infection. *Lane 3*: MRSA infection. Data are expressed as the mean + SEM. $^{**}P < 0.01$ compared with the control group (*lane 1*)

and CFU of the wounds in this animal model revealed no significant difference between MSSA and MRSA infections. This staphylococcal infection model should also prove useful in assessing the sensitivity of antibiotics against MRSA and MSSA in vivo.

Although PI is a commonly used, effective anti-microbial agent [16], it has cytotoxic effects for fibroblasts and keratinocytes at a concentration of more than 1 mg/ml in vitro [20]. Cadexomer-iodine is a commercially available ointment, which releases iodine slowly from beads of dextrin and epichlorhydrin, thereby reducing the cytotoxity of PI and providing effective debridement of necrotic tissue [5, 9, 29] and biofilm [2,3], and showing stimulation of epidermal regeneration [15]. A liposomal hydrogel containing 3% PI, another commercially available topical reagent, successfully cleans the wound, contains the infection and may even down-modulate inflammation and enhance wound closure [19]. The topical application of the mixture of sugar and PI has been reported to be useful for promoting healing in various cutaneous wounds, including burns and chronic ulcers, in clinical trials [4, 14, 17, 18, 22, 25]. Application of the mixture of sugar and PI has been shown to be different from that of sugar alone in terms of its efficacy in healing wounds. In our previous study with rabbits in vivo, we demonstrated that application of SP accelerated reepithelialization and increased the amount of granulation tissue and the number of capillaries significantly, whereas 70% sugar accelerated reepithelialization only [21]. On the other hand, by using *db/db* mice model in vivo, we demonstrated that the paste consisting of 70% sugar and the base did not significantly change any markers, including reepithelializaion, granulation tissue formation and capillary numbers, whereas SP significantly increased the amount of granulation tissue and capillary numbers in the wound compared to the no treatment group [21], suggesting that the base of SP had no effect on the same *db/db* mice model in the present study. In another previous report of an in vitro study, we demonstrated that SP and PI up-regulated intra- and extracellular urokinase-type plasminogen activator (u-PA) levels, transforming growth factor (TGF)-α production in keratinocytes, and the expression of extracellular matrix receptor integrins such as α1, α2, α3, α4, α5 and β1 in both keratinocytes and fibroblasts, whereas SP and sugar alone accelerated collagen synthesis in fibroblasts [20].

The mixture of sugar and PI is also thought to have an antimicrobial antibiotic effect, the sugar's hygroscopic effect reducing edema in wounds, while simultaneously carrying PI deep into the wound or ulcer, thereby eliminating deeper contamination [14]. In the present study with *db/db* mice, we demonstrated that the wounds infected with MRSA and treated with SP displayed good repair rates compared to those of non-treated mice in that reepithelialization was accelerated and CFU of MRSA was decreased. Invasive infections differ from wound contamination and colonization in that substantial amount of bacteria proliferate in the lesion, and erythema, exudation and pustules can be observed macroscopically. Also, numerous bacteria are cultured bacteriologically, and abscesses and bacterial components can be found in the ulcer histopathologically. In the present study, we confirmed these factors macroscopically and histopathologically (Fig. 1), as well as confirming CFU (Fig. 3c). In our experiment, we scrubbed the surface of the infected wound with a sterile swab, and soaked the swab in PBS. The solution was then cultured, and CFU was counted. Homogenizing the tissue of each wound would render more accurate, quantitative results; as we needed more sample tissues to observe the histopathology, we cultured the wound surface only. Although counting CFU by scrubbing the wound with a swab is not an ideal quantitative method, it is useful in estimating the extent of infection semi-quantitatively.

In conclusion, our animal model employing diabetic mice infected with MRSA or MSSA is a useful tool for investigating infectious skin ulcers. SP is also an effective topical agent for the treatment of diabetic ulcers with MRSA infection.

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